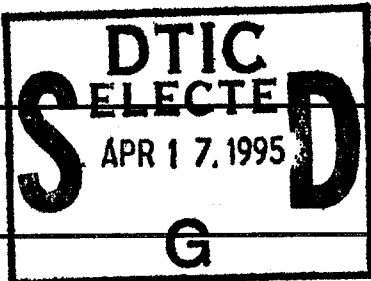


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FOREWORD


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Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

In conducting research utilizing recombinant DNA technology, the investigators adhered to current guidelines promulgated by the by the National Institutes of Health, including the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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ABSTRACT

Research has been directed at the development of a safe, inexpensive and effective hepatitis A vaccine. Neutralization epitopes on the surface of the HAV capsid have been characterized in detail. A single linear antigenic site on the virus capsid which found to be reactive with a murine neutralizing monoclonal antibody. Poliovirus antigenic chimeras were engineered which expressed amino acid sequences of this HAV epitope within its VP1 capsid protein, but were not neutralized by the cognate monoclonal antibody. Translational control elements contained within the 5' nontranslated region of the genome were mapped. Infectious HAV cDNA constructs were engineered which contain extensive deletion mutations within a pyrimidine rich region located immediately upstream of the internal ribosomal entry site. Deletion of up to 44 nts in this region did not alter the growth of the virus in BS-C-1 cells. However, extension of the deletion in a 3' direction by several additional nucleotides resulted in a marked ts phenotype. The attenuating effect of these mutations remains under study. Significant qualitative differences were demonstrated in anti-HAV antibodies present following administration of immune globulin vs. active immunization with inactivated vaccine, suggesting that the latter induces antibodies with relatively low affinity for the virus.

INTRODUCTION

Hepatitis A as a military hazard Hepatitis A is a potentially debilitating infectious disease which may reach epidemic proportions under poor sanitary conditions, and military history records many instances in which epidemics of this disease have substantially hindered the operational effectiveness of armed forces (Bancroft and Lemon, 1984). Because hepatitis A is less prevalent in the United States than in many other countries, the prevalence of antibody to hepatitis A virus (HAV) is very low among American soldiers. Relatively recent surveys suggest that less than 15% of American soldiers have naturally acquired anti-HAV (Bancroft and Lemon, 1984), indicating that the vast majority of soldiers are susceptible to hepatitis A. In the 1990s and beyond, American forces are most likely to encounter this virus when deployed overseas to developing regions where hepatitis A may be endemic. The risk of exposure to HAV is substantially magnified when previously existing public health facilities and sanitation practices are disrupted by military conflict, such as occurred within Kuwait during Operations Desert Shield and Desert Storm. This recent large scale mobilization of American forces reemphasized the risks and special problems posed by HAV to military operations. While short-term protection against hepatitis A was provided by timely administration of immune globulin (IG) prior to deployment, supplies of IG were rapidly exhausted. Readministration of IG is required at a minimum of 6 month intervals in order to maintain continued protection. Although this did not pose a problem during Desert Storm due to the relatively brief nature of the operation, this may be difficult to accomplish in the future with troops which remain deployed and engaged in action against hostile forces. There is thus an urgent need for development of a vaccine capable of providing safe, long-term, active immunity against HAV. It is important that such a vaccine be available to the military forces of the United States at reasonable cost.

Formalin-inactivated hepatitis A vaccines Following the successful development of a prototype, formalin-inactivated HAV vaccine produced in cell culture by Army investigators (Binn et al., 1986), formalin-inactivated HAV vaccines have been developed by several commercial vaccine manufacturers (for recent reviews, see Siegl and Lemon, 1990, and Lemon and Shapiro, 1994). A clinical study of an inactivated HAV vaccine produced by Merck & Co. (VAQTA™) was carried out in children in Monroe, NY, during 1991. This study demonstrated a high level of protection against symptomatic hepatitis A following administration of one dose of this vaccine (Werzberger et al., 1992). A similar study carried out in Thailand by U.S. Army investigators demonstrated a similar level of efficacy after two doses of an inactivated HAV vaccine produced by SmithKline Beecham (HAVRIX™) (Innis et al., 1994). While similar efficacy studies have not been carried out in adults, these vaccines appear to offer a level of protection that would be more than adequate for protection of military forces operating in hepatitis A endemic regions (Lemon, 1994).

However, several problems continue to hinder the use of these formalin inactivated HAV vaccines by the U.S. military. Neither of these vaccines is yet licensed by the Food and Drug Administration for use within the United States. In addition, available data suggest that multiple doses of formalin inactivated HAV vaccines will be required in adults in order to elicit even moderately long-lasting protective levels of immunity. Multiple-dose schedules, with late booster doses given at least 6 months after the first immunization may prove inconvenient for use in military populations. Furthermore, the costs of these vaccines will be high. This is due to the comparatively poor in vitro yields of antigen obtained with current vaccine virus strains, as well as the purification procedures required for production of an acceptable, modern vaccine. The current retail price of HAVRIX™ in Switzerland is approximately US\$ 48.00 per dose (Lemon and Shapiro, 1994). Although competition and government procurement practices are certain to result in lower purchase prices, high costs are likely to prohibit the universal use of inactivated vaccines among U.S. military forces for many years to come. Because of the uncertainties concerning the

future use of inactivated HAV vaccines by the U.S. military, work under this grant has focused on efforts to develop alternative approaches to development of HAV vaccines.

Synthetic immunogens for protection against HAV In previous work, we mapped an immunodominant neutralization antigenic site on the surface of the HAV capsid by analysis of murine monoclonal antibody-resistant neutralization escape variants of HAV (Ping et al., 1988; Cox et al., 1990; Ping et al., 1992), and by characterizing the competition between such monoclonal antibodies for binding to the virus capsid (Stapleton et al., 1987). These studies indicate that the β B- β C loops of capsid proteins VP3 and VP1 contribute to an immunogenic structure on the virus surface that dominates in the human immune response (Ping et al., 1988; Day et al., 1990). Although, this site is largely conformationally defined, we reasoned that short oligopeptide sequences representative of the relevant regions of VP3 and VP1 might be both antigenic and potentially immunogenic with respect to HAV neutralizing activity. Peptide immunogens are highly stable reagents; they are potentially very inexpensive and extremely safe inasmuch as they are chemically defined. We have previously shown that only low levels of neutralizing antibody are required for protection against hepatitis A (Stapleton et al., 1985), a fact which was recently confirmed during an efficacy trial with the Merck inactivated HAV vaccine Werzberger et al., 1992) (also see below). We thus postulated that peptide immunogens may have practical application to the prevention of hepatitis A.

We have taken several experimental approaches to this problem:

(1) We continued efforts to map the neutralization epitopes of HAV by the isolation and characterization of monoclonal antibody resistant neutralization escape HAV mutants. Studies examining the neutralization resistance phenotypes of these mutants, coupled with identification of the capsid protein mutations responsible for neutralization escape, resulted in the most complete mapping to date of epitopes recognized by murine and human antibodies to HAV (Ping and Lemon, 1992) (for details, see Annual Report No. 3).

(2) Based on the information provided in (1) above, we synthesized on polyethylene pins a large series of octapeptides representing the primary sequence of regions of the HAV capsid proteins VP3 and VP1 which the work with escape mutants described above suggested were involved in antibody recognition. We probed such peptides with monoclonal and polyclonal anti-HAV antibodies in peptide ELISAs (PEPSCAN) (Geysen et al., 1984, 1987). This approach resulted in the identification of a linear antigenic determinant within the HAV capsid structure which is recognized by a single neutralizing murine monoclonal antibody (H7-C27). Three different peptides representing this linear determinant were synthesized. However, antibodies raised to these peptides in either rabbits or guinea pigs neither neutralized nor immunoprecipitated HAV (see Annual Report No. 4).

(3) We characterized the antigenicity and immunogenicity of chimeric picornaviruses in which appropriate HAV peptide sequences replaced residues within an antigenic loop of capsid protein VP1 of the Sabin type 1 poliovirus (Lemon et al., 1992). These antigenic chimeras were constructed using an infectious poliovirus cDNA clone which contains a mutagenesis cassette in the region encoding VP1 (Burke et al., 1989). HAV/poliovirus chimeras have the potential of presenting HAV peptides in a conformationally constrained manner, and we were encouraged by preliminary success with such chimeric viruses (see Progress Report Nos. 1 and 2). During the current report period, we constructed additional poliovirus antigenic chimeras expressing segments of the H7-C27 linear epitope described in (2) above. These results are described in the narrative section below.

Improved candidate attenuated HAV vaccines In addition to exploring approaches to subunit HAV vaccines, we have continued efforts to better understand the molecular basis of the attenuation of HAV that frequently accompanies the adaptation of the virus to growth in cell

culture. This work follows that supported under a previous contract with the U.S. Army Medical Research and Development Command (DAMD17-85-C-5272). Previous attempts to develop an attenuated HAV vaccine have focused on several cell culture-adapted virus strains (for a review, see Lemon, 1985; Siegl and Lemon, 1990). However, such cell culture-adapted viruses appear to replicate poorly in primate liver, and have very poor immunogenicity in man. There is thus a need for less highly passaged cell culture-adapted variants, or for novel approaches to selecting different attenuated HAV vaccine candidates.

Although work by Emerson, Purcell and associates (Emerson et al., 1992) has shown that mutations in the P2 region of HAV are important in determining the cell culture growth properties of cell culture-adapted variants of HAV (Emerson et al. 1992), we demonstrated that mutations within the 5'NTR of cell culture adapted variants of the HM175 virus promote the growth of this virus in BS-C-1 but not FRhK-4 cells (Day et al., 1990; Day et al., 1992) (see Annual Report No. 4). These results suggest that the 5'NTR also plays an important role in the host range change which characterizes the attenuation phenotype of cell culture adapted variants of HAV. This has led us to reason that further directed mutagenesis within the 5'NTR of HAV may lead to attenuation phenotypes which may be exploited for development of novel candidate attenuated vaccine strains. Such an hypothesis is buttressed by ample evidence that the 5'NTRs of other picornaviruses may contain important attenuating mutations. We have shown that the HAV 5'NTR structure is very similar to that of the murine cardioviruses (Brown et al., 1991) (see below). Because cardioviruses with deletion mutations within the poly-C tract of the cardioviral 5' NTR may be highly attenuated in vivo, yet grow well in cell culture, (Duke et al., 1990), we suspected that HAV mutants with deletions within the first pyrimidine-rich tract of the 5'NTR (bases 95-152) might have interesting attenuation properties. Thus during the period covered by this report, we generated a substantial number of viable viral mutants with large deletion mutations within this region (Shaffer et al., 1994). These mutants are described in detail in the narrative section below. After characterizing these mutations within the context of the genetic background of cell culture-adapted virus, we have created similar mutations in a wild-type (virulent) genetic background. At present, in collaboration with Dr. Purcell's group at the National Institutes of Health, we have begun to assess the impact of one of these deletions upon the virulence of the virus in marmosets.

Immune response to inactivated HAV vaccine Recent clinical efficacy trials of formalin-inactivated HAV vaccines (Werzberger et al., 1992; Innis et al., 1994) have provided an opportunity to examine correlations between levels of antibody induced by immunization and clinical protection against disease. During the period covered by this report, we have carried out extensive studies of the immune response to the Merck inactivated HAV vaccine (VAQTA™), using sera collected from children during the study of vaccine efficacy carried out in Monroe, NY (Werzberger et al., 1992). These studies employed four different methods for detection of anti-HAV and have helped to define levels of anti-HAV antibody which are protective in children. Moreover, they have defined important differences in the anti-HAV antibodies which are present following active immunization with inactivated HAV vaccine and that present following passive immunization by administration of immune globulin. This work will be useful in guiding future studies of candidate HAV vaccines and is detailed in the narrative section which follows.

NARRATIVE SUMMARY OF RESEARCH PROGRESS

1. Poliovirus antigenic chimera expressing the H7-C27 epitope

As described in Report Nos. 3 and 4, the H7-C27 neutralizing monoclonal antibody to HAV epitope is unique in that it recognizes two discontinuous segments of VP1 which are each capable of functioning as linear antigenic determinants. All other anti-HAV monoclonal antibodies recognize conformational epitopes of HAV and fail to bind to synthetic peptides representing

regions of the viral capsid proteins which other evidence suggests are involved in the binding of neutralizing antibodies (Ping and Lemon, 1992). The amino terminal element of the H7-C27 epitope (with a core activity represented by the sequence "-Phe-Pro-Arg-Ala-Pro-Leu-Asn-", residues 1-259 through 1-265) appears to have greater antigenic activity than the carboxy terminal element ("-Ser-Met-Met-Ser-Arg-Ile-Ala-Ala-", residues 1-274 through 1-281) (see Annual Report No. 4).

As described in previous reports, we synthesized three peptides which represent segments of this putative antigenic site: peptides 4918 and 4919 represent the amino terminal element of the epitope (residues 1-256 to 1-268), with carboxy- and amino-terminal (respectively) Gly-Gly spacers and Cys residues for coupling to carrier. Peptide 4920 represents residues 1-256 to 1-283, with a carboxy terminal Cys residue for coupling to carrier. Preliminary studies utilizing solid-phase peptide ELISA assays suggested that H7-C27 (but not another monoclonal antibody, K2-4F2) weakly recognized peptides 4918 and 4919 (see Annual Report No. 3), but not peptide 4920. More recent results have failed to confirm the binding of H7-C27 to either peptide 4918 or 4919, despite the ability of the monoclonal to specifically recognize short octapeptide sequences included within the larger sequences of these peptides. As described in Annual Report No. 4, pairs of guinea pigs and rabbits were immunized with each of these peptides following their conjugation to keyhole limpet hemocyanin. This work was done in collaboration with Dr. John Cullen of the College of Veterinary Medicine of North Carolina State University. While good anti-peptide activity was demonstrated by PEPSCAN (in some cases matching the activity of the H7-C27 monoclonal antibody), none of these antisera contained detectable neutralizing or immunoprecipitating activity directed against the native virus (see Annual Report No. 4).

The discontinuous, complex nature of the H7-C27 epitope identified by PEPSCAN (see Figure 2 in Annual Report No. 4) suggests that the VP1 loop containing residues 1-256 through 1-283 may assume a dominant conformation in which part or all of the relatively hydrophobic sequence "-Ser-Asn-Ala-Met-Leu-Ser-Thr-Glu-" (residues 1-266 through 1-273) may be buried and not available for antibody binding. We considered the possibility that insertion of segments of the H7-C27 reactive sequence into the β B- β C loop (antigenic site 1) of the VP1 protein of Sabin type 1 poliovirus might impose conformational constraints which could improve its antigenicity and, potentially, its ability to elicit antibodies reactive with native HAV (Lemon et al., 1992). We thus constructed two chimeric polioviruses expressing H7-C27 reactive segments of the HAV VP1 protein, using the modified full-length Sabin type 1 poliovirus pCas-7 vector (obtained from Dr. J. Almond of the University of Reading). This vector contains unique *Sal*I and *Dra*I sites flanking the sequence encoding antigenic site 1 of poliovirus VP1, with a frame shift mutation placed between these sites which renders the viral sequence nonviable. The region between these restriction sites in pCas-7 was replaced with synthetic double-stranded oligonucleotide linkers which both corrected the frame-shift mutation and inserted the HAV sequences of interest. The accuracy of these plasmid constructions was confirmed by DNA sequencing. Infectious chimeric viruses were subsequently rescued from HeLa cells following transfection with RNA transcripts made from these recombinant plasmids. The HAV sequences inserted into antigenic site 1 of Sabin 1 virus are as shown overleaf (HAV sequences are underlined) for antigenic chimeras HAEP1-1 and HAEP2-2:

HAEP1-1
(p1 stock = 3×10^8 pfu/ml)

UAUAACCGUCGACAAC GAGUUUUUAUUUCCCCAGAGCUCCAUUGAACUCAAUGCC AAACU
I T V D N E F Y F P R A P L N S N A K L

HAEP2-2
(p1 stock = $0.5\text{-}1.2 \times 10^8$ pfu/ml)

UAUAACCGUCGACAAC UCCACUGAAUCAUGAUGAGCAGAAUUGCAGCUGGAGAC AAACUAUUU
I T V D N S T E S M M S R I A A G D K L F

The antigenicity of these new HAV/Sabin-1 chimeras has been examined in a preliminary fashion by plaque reduction neutralization using several different HAV-specific antibodies. Both chimeric viruses were neutralized by rabbit antisera raised to peptide 4920 (HAV VP1 residues 256-283, see Annual Report No. 4) (50% neutralization titers of 1:320 and 1:160 with rabbit 3279 antisera). This result confirms the correct expression of the peptide segment from HAV. However, neither chimeric virus was neutralized by the H7-C27 monoclonal antibody, nor by serum collected from an owl monkey either before or after infection with HAV. Thus, the results obtained with these two chimeric viruses resemble the results we previously obtained with peptides synthesized in bulk. The conclusion to be drawn from these studies at this point in time is that even the H7-C27 epitope is not easily modeled by synthetic peptides 13-28 residues in length, and that expression of the two discontinuous segments of the H7-C27 epitope within the antigenic site 1 surface loop of poliovirus VP1 does not enhance HAV-specific antigenicity. We do not understand why this neutralizing monoclonal antibody recognizes the smaller octapeptides employed in the PEPSCAN assay (see Annual Report No. 4), and plan to evaluate this further by construction of chimeric polioviruses containing these 8-residue sequences. Because plaque-reduction neutralization of a chimeric picornavirus may not be a good measure of its immunoreactivity (particularly with monoclonal antibodies) due to the potential requirement that the foreign epitope be oriented in a fashion which allows the bivalent binding of antibody across pentamer interfaces (Emini et al., 1983; Mosser et al., 1988), we plan to raise antisera to these two chimeric viruses in rabbits and test these antisera for neutralizing activity against HAV.

2. Construction and characterization of viable HAV mutants with large 5'NTR deletions

Although there are substantial differences between the predicted secondary structures of the 5'NTRs of HAV and all other picornaviruses, the secondary structure of the HAV 5'NTR more closely resembles that of the cardioviruses and aphthoviruses than the corresponding structure in rhinoviruses and most enteroviruses (Brown et al., 1991). Among other similarities, the 5'NTRs of hepatoviruses, cardioviruses, and aphthoviruses share the potential to form two or more pseudoknots in the region immediately downstream of the 5' terminal hairpin (Brown et al., 1991; Clarke et al., 1987; 20). Immediately downstream of these putative pseudoknots is a pyrimidine-rich sequence which consists of an almost pure polycytidylic acid tract in the cardioviruses and aphthoviruses. In the hepatoviruses, the corresponding region contains a mixture of uridylic acids and cytidylic acids (in a 24:14 ratio in the HM175 strain of HAV), with only two purines located within a 40 nt long, nearly pure polypyrimidine tract (pY1 domain, nts 99-138) (Fig. 1). There are other pyrimidine-rich tracts within the 5'NTR of HAV (Chang et al., 1993), but the pY1 domain is the longest and most prominent of these regions. Although considerable sequence heterogeneity exists within this domain among different human hepatoviruses (Brown et al., 1991), the general features of this domain are conserved among all strains of HAV. A striking aspect of the pY1 domain, which is unique to the HAV 5'NTR among all other picornaviral 5'NTRs, is the presence of tandem repeats of the sequence motif (U)UCC(C). Curiously, this motif closely resembles the core sequence of the "box A" motif of Pilipenko et al. (Pilipenko et al., 1992), which is present in a

conserved location in all picornaviruses, about 20-25 nts upstream of the initiator AUG, and which may play an important role in internal initiation of translation. Previous modeling of the secondary structure of the 5'NTR of HM175 virus predicted that the pY1 domain and the immediately adjacent sequence from nts 139-154 were likely to be single-stranded (Brown et al., 1991). During the current report period, we have carried out RNase mapping of the secondary structure of this region of the 5'NTR, and have constructed a series of mutant viruses with large deletions involving the pY1 domain (Shaffer et al., 1994). We have shown that the pY1 domain forms an ordered structural element downstream of the putative 5' pseudoknots of HAV, but that this ordered structure is not required for efficient replication in cultured cells as long the sequence between nts 140-144 is present. In contrast, an extended single-stranded region immediately downstream of the pY1 domain, which includes nts 140-144, is required for efficient replication of the virus at physiologic temperatures (Shaffer et al., 1994).

Secondary structure of the 5' 300 nts of the 5'NTR of HAV. Covariant nucleotide substitutions within the 5'NTRs of different strains of HAV predict double-stranded helices that are conserved in the secondary structure of the RNA (Brown et al., 1991). The presence of numerous covariant substitutions provided a high level of confidence in predictions of the structure of the 3' half of the 5'NTR, but only a single cluster of covariant substitutions (near the top of stem-loop IIIa) has been identified upstream of nt 330. Thus previous predictions of the structure in this region of the 5'NTR (Brown et al., 1991) (Fig. 1) were based almost entirely upon thermodynamic considerations.

To test the validity of these predictions, we determined the sites at which synthetic 5'NTR RNA was susceptible to cleavage by RNases which preferentially cleave single-stranded (RNase S1, RNase T1, RNase T2) or double-stranded (RNase V1) RNA. The synthetic RNAs utilized in these experiments represented the 5' 980 or 1108 nucleotides of the HAV genome and included 10 additional nucleotides at the 5' terminus which were derived from the vector. These experiments, the results of which are summarized in Figure 1, generally confirmed the predicted secondary structure. Each region within the 5' 303 nts of the 5'NTR was examined in at least two separate experiments. The most prominent single-strand specific RNase cleavage sites were located precisely in the predicted loop regions of stem-loops I, IIa, IIb and IIId, and at the 5' and 3' ends of the extended region flanking the pY1 domain (nts 96-98, and 135-152). The most prominent sites at which the double-strand specific RNase V1 cleaved the RNA were located within the stem of stem-loops IIb (nts 81-84) and IIId (nts 282-285). Other V1 cleavage sites were at nts 74-76, in a region between stem loops IIa and IIb which would be base-paired in the second predicted pseudoknot (Fig. 1).

Surprisingly, RNase V1 cleaved the RNA at multiple sites within the pY1 domain, despite previous predictions that this region should be single stranded. These V1 cleavage sites centered on five groups of cytidylic acids that occur as part of the repetitive (U)UUCC(C) motifs (Figs. 1), but V1 cleavage also occurred at uridylic acids located just downstream of the pY1 domain (nts 141, 142). Significantly, no single-strand specific enzymes cleaved the RNA within the region containing the five repetitive (U)UUCC(C) motifs (nts 99-130), although relatively strong single-strand cleavage sites flanked this domain. These results indicate that the pY1 domain does not exist as a randomly ordered single-stranded RNA segment, but that it possesses an ordered structure. The V1 cleavages in this domain may reflect helical stacking of the RNA, or possibly noncanonical hemiprotonated C-C base pairing (Gehring et al., 1993). Since hemiprotonated C-C base pairing is more likely to occur at acidic pH, we carried out V1 digestions over a pH range between 7.6 and 6.0. There was no enhancement of V1 cleavage at low pH, as might be expected if C-C base pairing were occurring. Parallel analysis of a different region of the 5'NTR confirmed that the enzyme was fully active at pH 6.0.

These experiments also provided indirect evidence for the existence of the two pseudoknots predicted to involve stem-loops IIa and IIb (Fig. 1). Strong stops for reverse transcriptase were

found to occur exactly at the 3' end of these predicted stem-loop structures. A similar strong stop was not present at the 3' end of the 5' terminal hair-pin (stem-loop I), although a much weaker stop was sometimes observed within a G-C rich region of this stem-loop (nts 26-28, Fig. 1). Although it is possible that the helical stems of stem-loops IIa and IIb are sufficiently stable to inhibit the progression of reverse transcriptase, the fact that a similar strong stop was not observed at the 3' end of stem loop I, which is longer, more G-C rich, and predicted to have a much lower free energy (Fig. 1) (Jacobson et al., 1984), suggests that stem loops IIa and IIb are further stabilized by their involvement in pseudoknots.

Deletion mutagenesis of the pY1 domain. Although the sequence within the pY1 domain is more variable than in any other region of the 5'NTR (Brown et al., 1991), all human hepatovirus strains studied thus far contain a pyrimidine-rich sequence in this region which is 21-40 nts in length. Each of these virus strains also preserves the repetitive (U)UUCC(C) motif, although the number of these motifs varies from strain to strain. To determine whether deletion mutations within and flanking the pY1 tract would impair replication, we constructed a full-length cDNA clone with a large deletion (p Δ 99-144) in this domain. This mutant was constructed within an infectious, full length cDNA clone of the HM175 strain of HAV, pG7/18fP2. This parental clone was constructed by replacing the small *SacI/EcoRI* fragment of pG3/HAV7 (Cohen et al., 1987; Day et al., 1992) (HM175/P35 virus sequence) with the corresponding cDNA fragment from a rapidly replicating, cytolytic variant (HM175/18f virus) (Lemon et al., 1991), essentially replacing the P2 region of HM175/P35 with that of HM175/18f virus (see Annual Report No. 4). The virus rescued from this parental clone (also called pP35-pY1) contains the pY1 sequence of cell culture adapted HM175/P35 virus (P35-pY1 virus). The p Δ 99-144 deletion mutant was subsequently used for construction of additional mutants with smaller deletions (Shaffer et al., 1994). Each of the deletion mutations was confirmed by dsDNA sequencing prior to RNA transcription and transfection into permissive BS-C-1 or FRhK-4 cells. Results of transfections at 35.5 °C or 31 °C are summarized in Figure 2.

In direct transfection/radioimmunofocus assays carried out at 35.5 °C, transfection of RNA derived from pP16-pY1, which contains the wild-type HM175 sequence in the pY1 domain, generated viral replication foci which were identical in size to those derived from RNA transcribed from the parental construct, (Shaffer et al., 1994). However, multiple transfections with p Δ 99-144 RNA at standard temperature conditions of 35.5 °C, in either FRhK-4 or BS-C-1 cells and including two blind passages of transfected cell harvests, never resulted in recovery of viable virus (Fig. 2). p Δ 99-144 DNA was sequenced completely within the manipulated region (nts 25-632). There were no changes from the parental sequence other than the expected 46 nt deletion. To determine whether a lethal mutation may have occurred elsewhere in the genome, the *BspEI/BamHI* fragment (nts 25-632) from p Δ 99-144 was replaced with the corresponding fragment from the viable mutant, pP16-pY1. As expected, RNA from the resulting clone generated replication foci that were identical in size to those of pP16-pY1. Thus, deletion of an extended sequence between stem-loops IIb and IIIa (Δ 99-144, Fig. 2) resulted in the absence of successful RNA transfection at physiologic temperature.

RNA derived from cDNA clones with smaller deletions in the pY1 domain proved to be infectious under these conditions (Fig. 2). However, two different replication phenotypes were observed among the rescued viruses (Shaffer et al., 1994). Viruses rescued from p Δ 99-115, p Δ 99-130, p Δ 99-134, p Δ 96-134, p Δ 96-137, and p Δ 96-139 produced replication foci which were similar in size to those of pP16-pY1. Thus, a 44 nt long deletion mutation which included the entire pY1 domain (Δ 96-139) resulted in no apparent impairment of virus replication. In contrast, virus rescued from p Δ 131-144 produced very small replication foci in radioimmunofocus assays carried out at 35.5 °C. The small replication focus size observed with this virus prompted an examination of its temperature sensitivity. Parallel titrations of Δ 131-144 virus in BS-C-1 cells at 31 °C and 37 °C demonstrated a difference of 1.8 log₁₀ rfu/ml in the titer of the working virus stock determined in radioimmunofocus assays carried out at these two temperatures (*ts* index), confirming that Δ 131-

144 virus had a temperature-sensitive (*ts*) replication phenotype (Table 1). In contrast, the *ts* index of P16-pY1 virus was $0.35 \pm 0.08 \log_{10}$ rfu/ml in multiple assays. Consistent with these results, replication foci of Δ 131-144 virus were nearly as large as those of P16-pY1 virus at 31 °C (Shaffer et al., 1994).

***ts* Phenotypes of viruses with pY1 deletions extending to nts 140-144.** Recognition of the *ts* phenotype of Δ 131-144 virus led us to reevaluate the infectivity of RNA transcribed from p Δ 99-144 and p Δ 116-144, both of which failed to generate infectious virus in transfections of FRhK-4 or BS-C-1 cells at 35.5 °C (Fig. 2). Repeat RNA transfections of FRhK-4 cells at 31 °C resulted in the rescue of viruses with marked *ts* phenotypes. The *ts* index of Δ 99-144 virus was $3.6 \log_{10}$ rfu/ml while that of Δ 116-144 virus was $1.9 \log_{10}$ rfu/ml (Table 1). Because the *ts* indices of the Δ 96-137 and Δ 96-139 viruses were 0.29 ± 0.04 and $0.40 \log_{10}$ rfu/ml respectively, similar to that of the parent P16-pY1 virus (Table 1), these results suggested that the 3' extension of the deletion to include nts 140-144 was responsible for the *ts* replication phenotype. Interestingly, although deletion of the region spanning nts 99-130 (Δ 99-130 and Δ 99-134 viruses, Table 1) had no significant impact on virus replication at 37 °C, the deletion of this region in association with the deletion of nts 131-144 resulted in a significant enhancement of the *ts* phenotype (compare the *ts* indices of the Δ 99-144 and Δ 131-144 viruses, 3.6 vs. $1.8 \log_{10}$ rfu/ml respectively, Table 1). Because the *ts* index of the Δ 116-144 virus was only 1.9, this enhancement of the *ts* phenotype was due primarily to deletion of the highly conserved first 2.5 (U)UCC(C) motifs located between nts 99 and 115.

In order to define more precisely the nucleotide deletions responsible for the *ts* phenotype, two additional mutant cDNA clones were constructed, p Δ 96-140 and p Δ 96-141. RNA transfections at 35.5 °C produced viruses with moderate *ts* phenotypes (Table 1). The *ts* index of Δ 96-140 virus was $0.73 \pm 0.17 \log_{10}$ rfu/ml, greater than that of the parent virus P16-pY1 ($0.35 \pm 0.08 \log_{10}$ rfu/ml) (Table 1). The *ts* index of Δ 96-141 virus was $>1.4 \log_{10}$ rfu/ml (1.5, >1.12 , and $>1.5 \log_{10}$ rfu/ml in three separate experiments). Thus, progressively greater *ts* indices were observed with viruses in which the pY1 deletion mutations extended in a 3' direction into the sequence spanning nts 140 to 144 (GUUGU). However, we do not yet know whether deletion of this sequence alone confers the *ts* phenotype. Although these *ts* viruses replicated much more efficiently at the permissive temperature, the replication foci of viruses with very large deletions (Δ 96-141 and Δ 99-144) were smaller than non-*ts* viruses (e.g. Δ 96-137) at 31 °C.

dsDNA sequencing of the cDNA region (nts 25-632) manipulated during mutagenesis of two of the *ts* cDNA clones (p Δ 131-144 and p Δ 116-144) documented only the expected deletion mutations. Replacement of this segment in the non-*ts* pP16-pY1 clone with the corresponding segment from p Δ 131-144 conferred the *ts* phenotype on the product virus, confirming that the reduced replication capacity at 37 °C was due to the engineered deletion and not to an adventitious mutation elsewhere in the genome. Equally important, the expected deletions were confirmed in the RNA sequence of each of the rescued viruses (except Δ 96-139 and Δ 96-140, which were not sequenced) by antigen-capture/PCR of virus, followed by dsDNA sequencing of the amplified product (Jansen et al., 1990) (Table 1). In no case was there reason to suspect that any of the rescued virus stocks had developed revertant or pseudorevertant mutations to compensate for the engineered deletions, since replication foci were numerous and similar in size on primary passage in direct transfection/radioimmunofocus assays.

The phenotype of individual mutants was the same following successful transfection of either BS-C-1 or FRhK-4 cells. BS-C-1 cells were consistently more difficult to transfect, but the replication foci of each of the rescued viruses was larger in BS-C-1 cells than in parallel transfections carried out in FRhK-4 cells (data not shown). This observation is consistent with the fact that each of these viruses contains cell-culture adaptation mutations at nts 152 and 203-204 which we have shown promote the replication of the virus in BS-C-1 but not FRhK-4 cells (Day et al., 1992). The phenotypes of the rescued viruses have remained stable for up to four passages as

judged by the size of replication foci in radioimmunofocus assays. Further evidence for the stability of the *ts* phenotype was provided by experiments in which BS-C-1 cells infected with *ts* variants ($\Delta 99-144$ and $\Delta 96-141$ virus) were maintained for up to 3 weeks at the nonpermissive temperature (37 °C), following an initial 24 hrs incubation at the permissive temperature (31 °C). Virus harvests prepared from these cells were subsequently tested in radioimmunofocus assays at the nonpermissive temperature in order to detect large focus revertants. No such revertants were isolated (data not shown), but we continue attempts in this direction.

Analysis of *ts* virus replication under one-step growth conditions. Although radioimmunofocus size is an accurate measure of the replication efficiency of HAV in cultured cells (Day et al., 1992), BS-C-1 and FRhK-4 cells were infected under one-step growth conditions in order to quantitate better differences in the kinetics of replication of different deletion mutants. At the permissive temperature (31 °C) in BS-C-1 cells, the replication of $\Delta 131-144$ virus (*ts* index = 1.8) was somewhat delayed compared with replication of the parental P16-pY1 virus or the large deletion mutant $\Delta 96-137$ (Fig. 3). The latter two viruses demonstrated similar replication kinetics, with virus yields approaching maximum by 72 hrs postinoculation. In contrast, maximum yields of $\Delta 131-144$ were not reached until 144 hrs postinoculation. This difference in replication kinetics was reflected also in the somewhat smaller size of $\Delta 131-144$ replication foci at 31 °C. The higher intracellular virus titer immediately after adsorption of $\Delta 131-144$ (time = 0), compared with the other two non-*ts* viruses, could represent delayed and nonsynchronous uncoating of virus, but this was not consistently observed in one-step growth experiments. In the experiment shown in Figure 3, this difference likely reflects a difference in m.o.i.

At the nonpermissive temperature (37 °C), replication of $\Delta 131-144$ virus was further delayed, with no increase over input virus noted until after 72 hrs postinoculation. Between 72 and 216 hrs, the increase in the titer of $\Delta 131-144$ virus paralleled that observed between 18 and 72 hrs at the permissive temperature. In contrast, there was no difference in the growth kinetics of P16-pY1 and $\Delta 96-137$ viruses at 31 °C and 37 °C, consistent with the low *ts* indices of these viruses (Table 1). The fact that the rate of intracellular accumulation of $\Delta 131-144$ virus between 72 and 216 hrs at the nonpermissive temperature paralleled the rate of accumulation between 12 and 150 hrs at the permissive temperature suggested that the *ts* phenotype of $\Delta 131-144$ might be due to a temperature-sensitive step occurring relatively early in the virus replication cycle. However, more recent studies suggest that the *ts* defect is present throughout the replication cycle, as shifting culture conditions to the permissive temperature early in the infection cycle (hrs 1-18) does not result in substantial increases in virus yield by 72 hrs (Shaffer and Lemon, manuscript in preparation). Additional one-step growth experiments confirmed that the replication of $\Delta 131-144$ virus was significantly delayed in comparison with P16-pY1 and $\Delta 100-131$ viruses at 35.5 °C in both FRhK-4 and BS-C-1 cells.

Contribution of P2 region mutations to the *ts* phenotype. All of the deletion mutants described above were constructed in a background which included the P2 genomic region of the rapidly replicating, cytopathic strain, HM175/18f. Thus, it was possible that the *ts* phenotype of the mutants described above might be derived in part from one or more of the numerous mutations present in the P2 region (Lemon et al., 1991). To address this possibility, the P2 region from the cell culture-adapted HM175/P35 variant (pHAV/7) (Cohen et al., 1987) was reintroduced into the *ts* cDNA clone p $\Delta 131-144$ to produce p $\Delta 131-144$ /P2P35. Virus rescued from p $\Delta 131-144$ /P2P35 RNA demonstrated a *ts* phenotype similar to $\Delta 131-144$ virus (data not shown), indicating that the *ts* phenotype was not codependent upon the presence of HM175/18f P2 region mutations. However, as expected, this virus replicated much more slowly than $\Delta 131-144$, requiring 2-3 weeks for demonstration of replication foci following RNA transfection even at the permissive temperature.

Nature of the *ts* defect. We compared the thermostability of the $\Delta 131-144$ virus with that of the P16-pY1 parent, in order to determine whether the reduction in titer of this *ts* strain at the nonpermissive temperature might reflect increased thermolability of virions due to altered

interactions between capsid proteins and genomic RNA. The infectious titers of the P16-pY1 and Δ 131-144 viruses were reduced to a similar extent following brief incubation at temperatures ranging from 50-60 °C (Shaffer et al., 1994). Thus the *ts* phenotype of Δ 131-144 virus is not related to reduced thermostability of the virus. Additional studies suggest that the *ts* phenotype is due to a defect in RNA replication (Shaffer and Lemon, manuscript in preparation). This conclusion is based on the fact that insertion of a *ts* mutation into 5'NTR reporter gene constructs did not lead to reductions in 5'NTR-directed translation in transfected cells which were incubated at the nonpermissive temperature. In addition, RNase protection experiments indicate that the *ts* defect is associated with a marked reduction and delay in positive-strand RNA replication which closely parallels the reductions and delay in formation of infectious HAV (Shaffer and Lemon, manuscript in preparation). These latter findings argue strongly against a defect in viral packaging, and are consistent with the fact that the *ts* defect in virus replication is not corrected by brief shifts to the permissive temperature at either the beginning or end of the infection period (Shaffer and Lemon, manuscript in preparation).

Deletion mutation involving stem-loop IIb. All of the deletion mutations described above were located between stem-loop structures predicted to flank the pY1 region (Fig. 1). To determine the impact of extension of these deletions in a 5' fashion into stem-loop IIb, an additional cDNA mutant (p Δ 93-134) was constructed. Compared with the viable p Δ 96-134 mutant, the deletion mutation in p Δ 93-134 extends in a 5' direction by an additional 3 nucleotides and includes the 3' terminal 2 nucleotides of stem-loop (pseudoknot) IIb (Fig. 1). Multiple transfections of FRhK-4 or BS-C-1 cells with RNA derived from p Δ 93-134, at either 31 °C or 35.5 °C failed to yield infectious virus (Fig. 2). In addition, a serendipitously discovered second-site cDNA mutant derived from the viable p Δ 99-134 mutant which had an additional, random mutation involving a G to U substitution at nt 85 also failed to produce infectious virus following RNA transfection (data not shown). The G to U substitution at nt 85 would be predicted to destabilize the putative pseudoknot involving stem-loop IIb (Fig. 1). These data suggest that retention of the secondary and possibly tertiary RNA structure in this region of the 5'NTR is essential for infectivity of the virus, and provide further indirect support for the proposed structural model.

Impact of 5'NTR deletion mutations on the virulence of HAV. The work described above was carried out with mutations created within the genetic background of a rapidly replicating, cell culture-adapted and almost certainly highly attenuated virus. This has allowed rapid characterization of the impact of these deletion mutations on the replication of the virus, but precludes immediate assessment of their potential attenuating effects. In order to determine the impact of these 5'NTR deletion mutations on the virulence of HAV, identical mutations must be created in the genetic background of a virulent HAV variant. Toward this end, we obtained infectious cDNA clones of HM175/wt (wild-type) and HM175/8Y viruses from Dr. Suzanne Emerson of Dr. Robert Purcell's laboratory at the National Institutes of Health. The 8Y clone contains the full-length wild-type sequence of HM175 virus with a single substitution mutation at nt 3889 (2B protein) which facilitates replication in cell culture. Virulent virus may be rescued from the 8Y clone by RNA transfection of FRhK-4 cells or by direct RNA injection into primate (marmoset) liver. In contrast, virus can only be rescued reliably from the wt cDNA clone by direct RNA intrahepatic injection. Thus far, two mutations have been created in the HM175/wt and HM175/8Y background: Δ 99-137 and Δ 131-144. The validity of the plasmid constructions was documented by DNA sequencing. However, virus was rescued only from the 8Y clones following transfection of FRhK-4 cells. Collaborative studies have been initiated with Dr. Emerson to determine whether 8Y Δ 99-137 virus remains capable of causing disease in marmosets following direct intrahepatic injection of synthetic RNA.

We are very encouraged and excited by the results obtained thus far with these 5'NTR deletion mutants. However, evaluation of these potential candidate vaccine strains will be a long and complex process, as both parenteral and oral (intragastic) routes of administration will need to be assessed. Whatever their outcome, these studies should lead to important new knowledge

concerning the function of the pY1 domain of the 5'NTR and the impact of the *ts* phenotype on HAV virulence in animal models of hepatitis A.

3. Serological response to inactivated HAV vaccine: protective levels of neutralizing and immunoprecipitating antibodies

Previous experience with immune globulin prophylaxis of hepatitis A suggests that the protection afforded by active immunization with formalin-killed virus vaccines is most likely due primarily if not entirely to the production of antibodies which are capable of neutralizing HAV (Lemon, 1985). Passive immunization with immune globulin at usual doses (0.02-0.06 ml/kg body weight) results in only low levels of serum neutralizing antibodies, and generally does not lead to seroconversion when paired serum samples are tested by commercially available competitive inhibition immunoassays (e.g., HAVAB-EIA™, Abbott Laboratories, N. Chicago IL) for antibodies to HAV (anti-HAV) (Stapleton et al., 1985). These data indicate that only very low levels of serum neutralizing antibodies are required in order to achieve a high level of clinical protection against disease (Binn et al., 1986). However, the minimal protective level of anti-HAV is not known. Attempts have been made to compare the antibody responses following active immunization with those present following administration of immune globulin (Ambrosch et al., 1991), but there are good reasons to suspect that these antibodies may be qualitatively different.

In order to gain a better understanding of the protective antibody response induced by formalin-inactivated hepatitis A vaccine, we analyzed the anti-HAV response in two cohorts of children who were immunized with a highly purified, formalin-inactivated CR326F' strain hepatitis A vaccine (VAQTA™) manufactured by Merck & Co. In a recent controlled clinical trial, this vaccine was shown to induce complete protection against clinically apparent hepatitis A (100% efficacy, 97-100% CI) within 3 weeks of administration of a single 25 antigen unit dose to children aged 1 to 16 years. During the period covered by this report, we have examined in detail the antibody response in children immunized with the Merck vaccine. This study was carried out in collaboration with Merck investigators, and used several different methods for detection of anti-HAV: conventional solid-phase immunoassay (modified HAVAB) in comparison to an international standard, two different assays for measurement of HAV-neutralizing antibodies, the radioimmunofocus inhibition test (RIFIT) and HAV antigen reduction assay (HAVARNA), and a novel radioimmunoprecipitation assay (RIPA) for detection of anti-HAV (see Annual Report No. 4).

Serum specimens were collected from children enrolled in two separate trials of inactivated HAV vaccine (VAQTA™). MSD-020 was a multi-center open-label study of the immunogenicity and tolerability of vaccine administered to children 4-12 years of age. A subset of 50 children who had been enrolled in this trial and who had received a 25 antigen unit dose of vaccine was randomly selected from one of the study sites. Sera collected from these children prior to and 4 weeks after administration of the first dose of vaccine were examined for the presence of neutralizing antibody. MSD-023 was a large, randomized, placebo-controlled clinical trial which demonstrated the ability of this vaccine to prevent clinically evident hepatitis A in children aged 2-16 years. A subset of 20 children who had received two doses of vaccine (25 antigen units each) separated by 6 months, and who had not experienced clinical signs or symptoms of hepatitis A, was randomly selected and sera collected from these children at weeks 0, 4, 24 and 28 were tested for the presence of neutralizing antibody to HAV. The anti-HAV antibody levels present in children following immunization with inactivated HAV vaccine were compared with antibody levels present in 20 healthy adult subjects 7 days after administration of a single intramuscular injection of immune globulin (Gamastan™, 0.06 ml/kg). This study (MSD-005) was directed by Dr. Karen Midthun of the Johns Hopkins University.

Serologic methods for detection of anti-HAV Sera collected from the subjects enrolled in these studies was assessed by four different methods for detection antibody to HAV:

Modified HAVAB™ radioimmunoassay for anti-HAV Antibody to HAV was measured using the commercial HAVAB™ (Abbott Laboratories, N. Chicago, IL) radioimmunoassay as directed by the manufacturer except that, instead of mixing 10 µl serum and 200 µl of ¹²⁵I anti-HAV, 100 µl of serum was mixed with 100 µl of ¹²⁵I anti-HAV. A positive result was determined by using the cut-off cpm obtained with the positive control included with the kit and an anti-HAV negative human serum pool. The level of anti-HAV was quantitated by interpolation against a standard curve constructed using serial two-fold dilutions of the World Health Organization's Reference Anti-HAV Immunoglobulin (5-320 mIU/ml). The minimal concentration of antibody which is detectable in this assay is approximately 10 mIU/ml.

Radioimmunofocus inhibition test for neutralizing antibody to HAV (RIFIT) This assay for serum neutralizing antibody is based on the ability of anti-HAV to inhibit the development of macroscopic foci of virus replication in cells overlaid with agarose, and is thus analogous to a plaque reduction assay (Lemon and Binn, 1983). Serial dilutions of serum [1:2, 1:8, 1:32, and 1:128 (MSD-020), or 1:8, 1:80, and 1:800 (MSD-023 and MSD-005)] were mixed with equal volumes of the virus suspension (HM175/18f virus) prior to inoculation onto nearly confluent cultures of BS-C-1 cells. Radioimmunofoci were enumerated after 6-7 days growth. Serum samples were considered positive for neutralizing antibodies if a 1:8 initial dilution of serum inhibited the development of ≥80% of the radioimmunofoci developing in the absence of serum. Neutralizing antibody titers were based on the calculated 80% radioimmunofocus reduction endpoint determined by linear regression analysis over the range of (initial) serum dilutions tested.

HAV antigen reduction assay for neutralizing anti-HAV (HAVARNA) HAVARNA assays were carried out as described previously with several minor modifications (Krah et al., 1991). The infectious inoculum was prepared from a stock of the P28 variant of CR326F strain HAV, derived by 10 further passages of the F' (P18) variant of CR326 virus through MRC-5 cells (VAQTA™ vaccine strain). Virus infections were allowed to proceed for a 7 day period prior to harvest and assay for HAV antigen. Paired pre-immune and immune serum samples were assayed in parallel dilutions, and the titer of neutralizing antibody was assigned to the highest (final) dilution of immune serum which reduced by 50% or more the accumulation of viral antigen in a parallel dilution of the pre-immune serum.

³H-HAV radioimmunoprecipitation assay for anti-HAV (RIPA) This assay measures the ability of antibodies present in a serum sample to immunoprecipitate metabolically labelled virus particles, and was carried out as described previously (Lemon et al., 1992) (see Annual Report No. 4). Briefly, virus (HM175/18f strain) was labelled by growth in the presence of ³H-uridine and purified by rate-zonal ultracentrifugation. Labelled virus (approximately 1000 cpm in 10 µl) was added to 100 µl of each of a series of dilutions of a serum sample (1:8, 1:80, and 1:800) prepared in RIPA buffer, vortexed briefly and placed at 4 °C overnight. Washed protein A-bearing staphylococcal cells (Pansorbin™, CalBiochem) were added and after a brief incubation at room temperature, reaction mixtures were centrifuged in a microfuge. The amount of radioactivity present in the pellet and supernatant fractions was determined by counting in a liquid scintillation counter. Based on results with antibody-negative sera, serum dilutions were considered positive for precipitating antibodies if they resulted in greater than 30% precipitation of the labelled virus [$\text{cpm}_{\text{pellet}} \geq 0.3 (\text{cpm}_{\text{pellet}} + \text{cpm}_{\text{supernatant}})$].

Modified HAVAB and HAVARNA assays were carried out at Merck & Co. under direction of Drs. Philip Provost and John Lewis, while RIFIT and RIPA tests were done at The University of North Carolina at Chapel Hill.

Neutralizing antibody response to inactivated HAV vaccine Sera collected from 70 children four weeks after administration of a single dose of inactivated HAV vaccine (VAQTA™, Merck & Co.) were tested for HAV-specific neutralizing antibody activity. These children were participants in two separate clinical studies, MSD-020 (a vaccine immunogenicity study) and MSD-

023 (the Monroe efficacy study) (Werzberger et al., 1992). The geometric mean titers (gmt) of anti-HAV antibodies detected by modified HAVAB in these study groups were 49.3 and 45.7 mIU/ml, respectively. The results of the radioimmunofocus inhibition test (RIFIT) and the HAV antigen reduction test (HAVARNA) are summarized in Table 2. Thirty-four of 70 (49%) sera contained neutralizing antibodies detectable by RIFIT ($\geq 80\%$ reduction in focus number at a 1:8 serum dilution), while 56 of 57 (98%) tested sera contained neutralizing antibodies detectable in the HAVARNA assay ($\geq 50\%$ antigen reduction at a 1:4 serum dilution). Thus, under these conditions, the HAVARNA assay was more sensitive than the RIFIT assay for detection of the early neutralizing antibody response to HAV vaccine. This difference in sensitivity reflected the more stringent criterion for positivity in the RIFIT assay, and was matched by differences in the gmt of neutralizing antibodies detected by these two methods. By RIFIT, the reciprocal gmt for neutralizing antibody was 12.4 and 23.5 for the MSD-020 and MSD-023 vaccine recipients, respectively, compared with 61.0 and 93.9 by HAVARNA (Table 2) (gmts calculated only for positive serum samples). Matched preimmunization sera were tested by RIFIT only (data not shown). Of these, only 1 of 70 (1.4%) tested positive in this neutralizing assay (RIFIT titer = 1:8).

Neutralizing antibodies to HAV following booster immunization To assess the neutralizing antibody response to inactivated HAV vaccine following booster immunization, we tested additional sera collected from the 20 MSD-023 study participants before and 4 weeks after a second 25 antigen unit dose of vaccine administered 24 weeks (mean 220 days) following primary immunization (Table 3). The pre- and post-booster gmts of anti-HAV determined by modified or standard HAVAB were 56.3 and 10,300 mIU/ml, respectively. These results suggest that there was no reduction in antibody levels between 4 and 24 weeks following primary immunization and indicate a substantial anamnestic response to booster immunization. Similar results were obtained in the neutralizing antibody assays. By RIFIT, 12 of 20 (60%) of study subjects were positive for neutralizing anti-HAV (reciprocal gmt 21.2 for positive subjects) at week 24, while all 20 (100%) subjects were positive 4 weeks after the booster dose (reciprocal gmt > 543 , with 16 of 20 subjects having titers ≥ 800). By HAVARNA, each of the 20 (100%) subjects was positive for neutralizing antibodies before and after the booster immunization (reciprocal gmts 79.3 and 37,300, respectively) (Table 3).

A comparison of RIFIT and HAVARNA titers in individual serum samples indicated a generally low level of correlation when only 4 and 24 week sera were examined in a combined analysis of the MSD-020 and MSD-023 studies. This reflects considerable variance in results obtained in repeat assays of individual serum samples by either assay, and the fact that these early postimmunization sera contain relatively low levels of neutralizing antibodies which are near the limits of detection in these assays. In contrast, a much stronger correlation between RIFIT and HAVARNA assays was evident when post-booster sera from MSD-020 were included in this analysis ($R^2 = 0.8027$ by power regression). Similarly, a strong correlation was evident between titers obtained in either virus neutralization assay and the antibody content of serum as determined by quantitative HAVAB (Fig. 4). Both neutralization assays were able to routinely detect antibody concentrations below 100 mIU/ml (the approximate detection limit for the standard HAVAB test) in serum samples collected 4 and 24 weeks after immunization. As shown in Fig. 4a, all of the post-immunization serum samples which were negative by RIFIT (titer $< 1:8$) contained < 100 mIU/ml antibody by modified HAVAB.

Comparison of neutralizing antibody titers present following immunization with inactivated HAV vaccine and administration of immune globulin Immune globulin administered intramuscularly in a dose of 0.06 ml/kg is known to produce highly effective, short term protection against clinical hepatitis A. This protection is due exclusively to the presence of circulating passively administered antibodies to HAV. Thus, it was of interest to compare the levels of antibodies present following immunization with inactivated vaccine with those present shortly after administration of immune globulin. As shown in Table 2, the level of antibody detectable by modified HAVAB 7 days after administration of immune globulin to a group of 20

adults (MSD-005 study) was similar to that present in both groups of children 4 weeks after immunization with inactivated vaccine (46.8 mIU/ml, vs. 49.3 and 45.7 mIU/ml respectively). However, all 20 (100%) of the immune globulin recipients were positive for neutralizing antibodies by RIFIT, compared with only 34 of 70 (49%) vaccine recipients. These results suggest that there may be qualitative differences in the antibodies present shortly after passive and active immunization, with greater levels of virus neutralizing activity associated with similar antibody levels measured quantitatively by modified HAVAB radioimmunoassay following administration of immune globulin.

Detection of vaccine-induced antibodies to HAV by immunoprecipitation of ^3H -labelled virus A subset of the serum samples collected prior to and 4 weeks after immunization from children enrolled in MSD-020 were tested for anti-HAV activity by RIPA at 1:8 and 1:80 dilutions. In general, a lower percentage precipitation value was obtained at the 1:80 dilution with most pre-immunization serum samples, reflecting less nonspecific precipitation of the labelled virus (see Annual Report No. 4). In contrast, dilution had an opposite effect with serum collected postimmunization. Generally higher precipitation values were obtained at 1:80 compared with 1:8 serum dilutions. To establish criteria for positivity in this assay, a panel of paired sera from the MSD-020 study were tested by RIPA at both 1:8 and 1:80 dilutions. When preimmunization sera ($n = 40$) were tested at a 1:8 dilution, the mean percent label precipitated was 7.98 ± 4.66 S.D. At a 1:80 dilution, the mean percent label precipitated with the preimmunization sera ($n = 38$) was 5.98 ± 4.08 S.D. Comparable results with paired serum samples collected 4 weeks after immunization were 43.0 ± 19.3 S.D. (1:8 dilution) and 66.0 ± 16.4 S.D. (1:80 dilution). Based on these results, serum samples capable of precipitating $>30\%$ of label at either 1:8 or 1:80 serum dilutions were considered positive for HAV-specific precipitating antibodies. Using this conservative criterion, each of the preimmunization sera tested negative by RIPA, while 37 of 38 MSD-020 postimmunization sera were positive, 26 of 38 (68%) at 1:8 dilutions, and 37 of 38 (97%) at 1:80.

Similar results were obtained when serum samples collected at weeks 0, 4, 24, and 28 from the 20 children enrolled in the MSD-023 study were tested for the presence of precipitating antibodies by RIPA (Table 3). To better quantitate the RIPA response, postimmunization sera were tested at 1:8, 1:80, and 1:800 dilutions. At the 1:8 dilution, 11 of 20 (55%) serum samples collected 4 weeks after immunization while 18 of 20 (90%) samples collected 6 months after immunization were positive for antibody ($>30\%$ precipitation). This difference correlates with a slight increase in antibody content between weeks 4 and 24 as determined by modified HAVAB (gmt 45.7 vs. 56.3), and suggests that there is significant maturation of the antibody response during this period in some subjects despite a slight decline in the gmt of antibody determined by both RIPA and HAVARNA (Table 2). There was substantial variation in the percent virus precipitated by paired sera collected from individual subjects at 4 and 24 weeks: 10 subjects showed an increase, while 7 subjects had little change and 3 demonstrated decreases in the percent virus precipitated at the 1:8 dilution. Following the booster immunization at week 24, sera from all 20 children were strongly positive by RIPA at all three dilutions tested (gmt $> 1:800$). Since lower precipitation values were obtained at 1:80 (compared with 1:8) dilutions when preimmunization sera from the MSD-020 study were tested by RIPA (see above), preimmunization sera from MSD-023 subjects were tested only at a 1:8 dilution in an effort to conserve the labelled antigen. At this dilution, none of the MSD-023 sera precipitated $>30\%$ of the virus.

Correlation of RIPA titers with serum neutralizing antibody and mIU/ml content of sera determined by modified HAVAB The titers of precipitating antibody determined by the semi-quantitative RIPA (1:8, 1:80, and 1:800 dilutions) correlated well with other measures of anti-HAV response following administration of inactivated HAV vaccine. Thirteen of 40 sera collected either 4 or 24 weeks after a single dose of vaccine in the MSD-023 study had low RIPA activity (titer 1:80). Only 3 of these 13 sera (23%) tested positive by RIFIT, while 22 of 27 sera with high RIPA activity (titer $\geq 1:800$) had RIFIT titers $\geq 1:8$ (Table 4). Similarly, compared with those sera which had low RIPA activity, sera with high RIPA activity had higher neutralizing antibody titers

determined by HAVARNA (reciprocal gmt 147 compared with 35.6), and greater antibody content determined by modified HAVAB (65.3 vs. 29.8 mIU/ml). High serum neutralizing antibody and HAVAB titers following booster immunization at 24 weeks correlated with RIPA titers $\geq 1:800$ in each of the 20 subjects tested (Table 2). In addition, booster immunization resulted in an increase in the mean percent virus precipitated at each of the serum dilutions tested.

RIPA activity following administration of immune globulin Sera collected from adult subjects enrolled in the MSD-005 study 7 days after administration of immune globulin (Table 1) were tested for the presence of precipitating antibodies by RIPA at 1:8 and 1:80 dilutions. Surprisingly, only 8 of 20 (40%) sera were positive, and none of these sera had RIPA titers $>1:8$ (Table 2). Sera collected following administration of immune globulin had much lower RIPA activity than sera collected 4 weeks after administration of inactivated HAV vaccine, even though globulin recipients had comparable titers of antibody measured by modified HAVAB (compare Figs. 5A and 5B) and somewhat higher serum neutralizing antibody titers measured by RIFIT (compare Figs. 5C and 5D). These results indicate that there are significant qualitative differences in circulating anti-HAV antibodies present following passive and active immunization, despite similar quantitative results in solid-phase immunoassays and assays for serum neutralizing antibodies. To determine whether the cold ethanol fractionation method utilized for the isolation of immunoglobulin from plasma pools during the manufacture of immune globulin might be responsible for loss of activity in the RIPA assay, we directly assayed two separate lots of immune globulin (GAMMARTM, Armour) for RIPA activity. Immunoglobulins present in both lots efficiently precipitated labelled virus at concentrations between 3 and 300 $\mu\text{g/ml}$. Thus the absence of immunoprecipitating antibodies in recipients of immune globulin reflects the fact the concentration of antibody present in serum is insufficient for detection by RIPA despite the presence of appreciable serum neutralizing activity as well as modified HAVAB positivity.

These seemingly discrepant results strongly suggest that there are qualitative differences between the HAV-reactive antibodies present following passive and active forms of immunization against hepatitis A. Antibodies present 4 and 24 weeks after immunization with inactivated vaccine have high RIPA activity relative to virus neutralizing (or modified HAVAB) activity, in sharp contrast to antibodies present following administration of immune globulin which have very low RIPA activity relative to virus neutralizing (or modified HAVAB) activity. This difference is likely to reflect substantially lower affinity of antibodies induced by active immunization with respect to their ability to bind virus. The RIPA assay employs a large quantity of virus and should be biased toward detection of low affinity antibodies, while virus neutralization assays use many orders of magnitude lower concentrations of virus and are likely to be biased toward detection of high affinity antibodies. Similarly, the solid-phase HAVAB immunoassay is likely to be biased toward detection of high affinity antibody, as it entails the direct competition of antibody present in test sera with labelled, presumably high affinity, detector antibody.

Recognition of qualitative differences in the antibodies present following these two forms of immunization will be important in future studies of the immune response to HAV vaccines. While there is no question of the clinical efficacy of these vaccines in children (Werzberger et al., 1992; Innis et al., 1994), the results of these efficacy trials will have to be extrapolated to adults as similar efficacy studies have not been done in adult populations. Such extrapolation is best done on the basis of a comparison of the antibody responses in pediatric and adult populations, and this requires a complete understanding of the serologic measures employed. While the relatively low affinity of vaccine-induced antibody might suggest differences in the antigenicity of native and formalin-inactivated viruses, we have been unable to demonstrate such differences using a large panel of monoclonal antibodies in other studies. It is more likely that these differences reflect the low antigen mass to which vaccine recipients are exposed. Additionally, it is possible that booster immunization might lead to antibodies of higher affinity due to maturation of the immune response, but this has yet to be demonstrated.

CONCLUSIONS

The research supported under this grant during this reporting period has continued to focus on improved methods for the control of hepatitis A among military forces, including (1) approaches to development of subunit and peptide vaccines for hepatitis A, (2) the construction of novel, genetically engineered, attenuated HAV vaccine candidates, and (3) a detailed comparison of the serologic response to a commercially prepared, formalin-inactivated HAV vaccine.

During the past year, we constructed and evaluated two additional poliovirus antigenic chimeras which express regions of the HAV capsid that are recognized specifically as short synthetic peptides by an anti-HAV neutralizing monoclonal antibody (H7-C27). Neither of these chimeric viruses were neutralized by this monoclonal suggesting that they do not display the HAV peptide sequences in a relevant conformation. The immunogenicity of these chimeras must be assessed before their potential usefulness is known, but these findings, when taken in the context of previous results obtained under support of this grant, suggest that all of the important neutralization antigenic sites of HAV are exquisitely dependent upon quaternary interactions which occur between the capsid polypeptide chains during virus assembly. It does not appear likely that a useful HAV vaccine can be developed by subunit approaches including the use of synthetic peptides or picornaviral antigenic chimeras.

We have developed a very sensitive and specific immunoprecipitation method for detection of antibodies to HAV in subjects immunized with inactivated HAV vaccines. This radioimmunoprecipitation assay (RIPA) has been compared with much more labor-intensive virus neutralization assays and with a solid-phase competitive inhibition immunoassay. When applied to recent recipients of inactivated HAV vaccine, the RIPA test is substantially more sensitive than either of these other test methods. However, RIPA is very insensitive for detection of antibodies in recipients of immune globulin, even though such recipients have equal or greater serum HAV neutralizing activity. These results indicate the existence of important qualitative differences in the antibody responses following active vs. passive immunization which will be important to consider in the evaluation of future clinical trials of new and existing HAV vaccines.

Over the past several years, we have completed a detailed examination of the structure and function of the 5'NTR of HAV, including its role in cap-independent internal initiation of HAV translation and the characterization of cellular proteins which bind specifically to this region of the viral RNA. This work is fundamental to the construction of novel attenuated vaccine candidates which may replace existing attenuated candidate vaccines which have demonstrated weak immunogenicity and a requirement for parenteral inoculation in multiple clinical trials. During the current report period, we have exploited our understanding of the structure and function of the 5'NTR to construct a series of mutant viruses with large deletions involving the pY1 domain of the 5'NTR. We have shown that the pY1 domain forms an ordered structural element downstream of two pseudoknots present near the 5' terminus of HAV RNA, and have demonstrated that this ordered structure is not required for efficient replication in cultured cells as long the sequence between nts 140-144 is present. In contrast, an extended single-stranded region immediately downstream of the pY1 domain, which includes nts 140-144, is required for efficient replication of the virus at physiologic temperature. These studies have led to the construction of several novel HAV mutants, some of which have a strong *ts* phenotype and which may have unique attenuation properties *in vivo*. The first of several potential attenuated vaccine candidates is now being evaluated in a primate model of hepatitis A in collaboration with investigators at the National Institutes of Health.

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Appendix

Table 1. Temperature sensitivity of 5'NTR deletion mutants

Virus	Radioimmunofocus Size		<i>ts</i> index ¹	RNA Sequencing ²
	31°C	37°C		
P16-pY1	+++	+++	0.35 ± 0.08	Yes
P35-pY1	+++	+++	0.22	Yes
Δ99-115	+++	+++	0.11	Yes
Δ99-130	+++	+++	0.54	Yes
Δ99-134	+++	+++	0.21	Yes
Δ96-134	+++	+++	n.d. ³	Yes
Δ96-137	+++	+++	0.29 ± 0.04	Yes
Δ96-139	+++	+++	0.40	n.d.
Δ96-140	++(+)	+	0.73 ± 0.17	n.d.
Δ96-141	++(+)	+	>1.40	Yes
Δ99-144	++	(+)	3.60	Yes
Δ116-144	++(+)	(+)	1.90	Yes
Δ131-144	++(+)	+	1.80	Yes

¹*ts* index = \log_{10} [titer 31°C] - \log_{10} [titer 37°C] in radioimmunofocus assays carried out in BS-C-1 cells, ± S.E. where 3 or more assays were carried out. The Δ96-139 result is a mean of two assays, and the Δ96-141 result a mean of 3 assays (see Shaffer et al., 1994).

²Mutation confirmed by RNA sequencing of rescued virus.

³n.d. = not done.

Table 2. Measurement of antibody to HAV in adult recipients of ISG (7 days) and pediatric subjects 4 weeks after a single dose of MSD vaccine

	MSD-020 Vaccine Study		MSD-023 Vaccine Study		MSD-005 ISG Study	
	pos/n	gmt	pos/n	gmt	pos/n	gmt
Modified HAVAB (mIU/ml)*	50/50 (100%)	49.3	20/20 (100%)	45.7	20/20 (100%)	46.8
Radioimmunofocus inhibition (RIFIT) (80% inhibition at 1:8 serum dilution)	22/50 (44%)	12.4	12/20 (60%)	23.5	20/20 (100%)	27.5
HAV antigen reduction (HAVARNA) (50% inhibition at 1:4 serum dilution)	36/37 (97%)	61.0	20/20 (100%)	93.9	n.d.	n.d.
³ H-HAV immunoprecipitation (RIP) (30% precipitation at 1:8 or 1:80 dilution)	42/43 (98%)	n.d.	20/20 (100%)	>400	8/20 (40%)	8

*positive considered $\geq 10\text{mIU/ml}$

Note: gmt values calculated only for positive samples: RIFIT $\geq 1:8$, HAVARNA $\geq 1:4$, RIP $\geq 1:8$.

Table 3. Measurement of antibody to HAV in MSD-023 study subjects at 4, 24, and 28 weeks

	Week 4 (mean 37.3 days)		Week 24 (mean 220 days)		Week 28 (mean 253 days)	
	pos/n	gmt	pos/n	gmt	pos/n	gmt
Modified HAVAB (mIU/ml)*	20/20 (100%)	45.7	20/20 (100%)	56.3	20/20 (100%)	10,300
Radioimmunoassay inhibition (RIFIT)	12/20 (60%)	23.5	12/20 (60%)	21.2	20/20 (100%)	>543
HAV antigen reduction (HAVARNA)	20/20 (100%)	93.9	20/20 (100%)	79.3	20/20 (100%)	37,300
³ H-HAV immunoprecipitation (RIP)	20/20 (100%)	>400	19/20 (95%)	>343	20/20 (100%)	>800

*positive considered $\geq 10\text{mIU/ml}$ Note: gmt values calculated only for positive samples: RIFIT $\geq 1:8$, HAVARNA $\geq 1:4$, RIP $\geq 1:8$.

Table 4. Serum Neutralizing Antibody and Modified HAVAB Assay Activity in Sera with Various Levels of RIPA Activity¹

RIPA Titer	n	Modified HAVAB % positive (gmt)	RIFIT % positive (gmt ²)	HAVARNA % positive (gmt)
<8	1	100% (16 mIU/ml)	0%	100% (1:8)
1:80	13	100% (29.8 mIU/ml)	23% (1:5.2)	100% (1:35.6)
≥800	27	100% (65.3 mIU/ml)	81% (1:17.2)	100% (1:147)

¹Table includes results only from week 4 and 24 sera from MSD-023 study subjects.

²For calculation of gmt, titers <1:8 were assigned a value of 4.

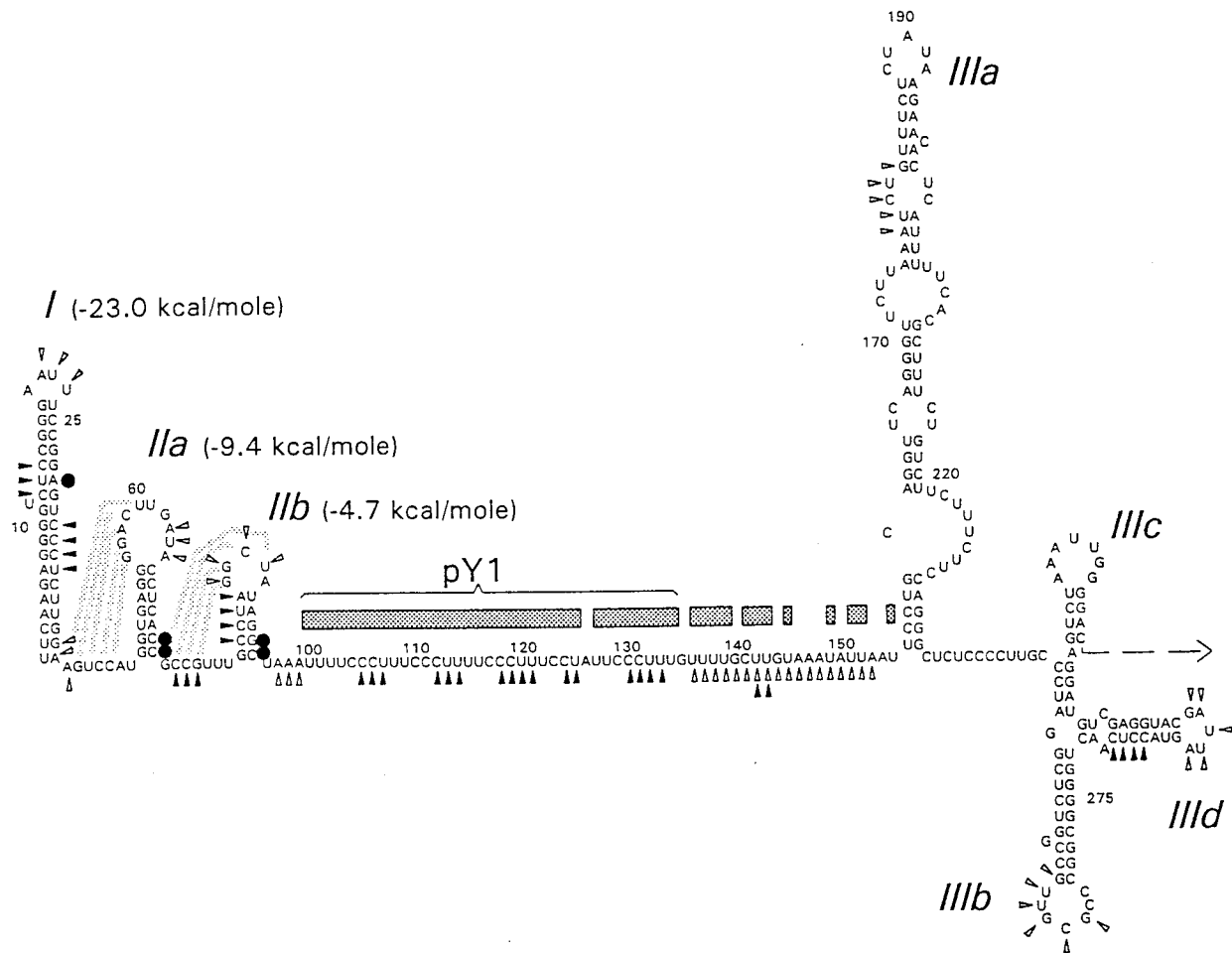


FIGURE 1. Secondary structure proposed for domains I, II, and III of wild-type HM175 5'NTR RNA, showing sites of cleavage by single strand specific (T1, T2, and S1) nucleases (open arrow heads) and cobra venom RNase V1 (solid arrow heads). Strong primer-extension stops are marked by large dots. The thermodynamic stability of stem loops I, IIa, and IIb was calculated by the use of the RNAFOLD program (PC/Gene, Intelligenetics), and does not include consideration of possible pseudoknot interactions. Pyrimidine nucleotides within the extended domain separating stem loops IIb and IIIa (nts 95-154) are marked with a shaded bar.

	90	100	110	120	130	140	150	160		
Wild-type	UAGGCUAAA	UUUCCCUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	31 °C	35.5 °C
	□□□□		□□□□		
pP16-pY	UAGGCUAAA	UUUCCCUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	+	+
pP35-pY	UAGGCUAAA	UUUCCCUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	n.d.	+
pΔ99-115	UAGGCUAAA	-----	-----	-----	-----	-----	-----	-----	n.d.	+
pΔ99-130	UAGGCUAAA	-----	-----	-----	-----	-----	-----	-----	n.d.	+
pΔ99-134	UAGGCUAAA	-----	-----	-----	-----	-----	-----	-----	n.d.	+
pΔ96-134	UAGGCU	-----	-----	-----	-----	-----	-----	-----	n.d.	+
pΔ93-134	UAG	-----	-----	-----	-----	-----	-----	-----	-	-
pΔ96-137	UAGGCU	-----	-----	-----	-----	-----	-----	-----	n.d.	+
pΔ96-139	UAGGCU	-----	-----	-----	-----	-----	-----	-----	n.d.	+
pΔ96-140	UAGGCU	-----	-----	-----	-----	-----	-----	-----	n.d.	+
pΔ96-141	UAGGCU	-----	-----	-----	-----	-----	-----	-----	n.d.	+
pΔ99-144	UAGGCUAAA	-----	-----	-----	-----	-----	-----	-----	+	-
pΔ116-144	UAGGCUAAA	UUUCCCUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	+	-
pΔ131-144	UAGGCUAAA	UUUCCCUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	CCCUUUU	+	+
									
						ts				

FIGURE 2. pY1 deletion mutations constructed within a full-length cDNA clone of the HM175 virus. The wild-type virus sequence is shown at the top, with mutant plasmids listed at the left: () nucleotides predicted to be involved in base-pairing interactions in stem-loops IIb (nts 90-94) or IIIa (nts 155-159); (•) positions cleaved by single-strand specific RNases (see Fig. 1). Direct transfection results at 31°C and 37°C are shown at the right: (+) replication foci demonstrated; (-) no replication foci demonstrated; n.d. = not determined. Nucleotides 140-144 which are deleted in *ts* viruses are indicated at the bottom.

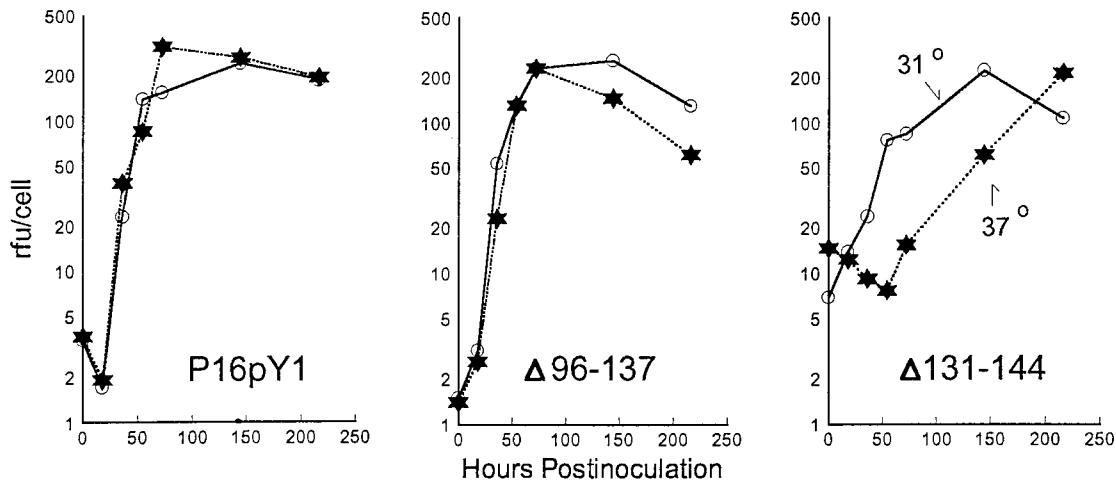


FIGURE 3. Intracellular virus accumulation under one step growth conditions at 31°C or 37 °C. Panels: (A) P16-pY1 virus, (B) Δ96-137 virus, (C) Δ131-144 virus. BS-C-1 cells were infected at an m.o.i. of 4 infectious particles per cell and then incubated at either 31°C (—) or 37 °C (---). At the indicated time points, monolayers were washed and lysed by the addition of 0.1% SDS. Virus titers in lysates were determined by radioimmunofocus assay in BS-C-1 cells at 31 °C.

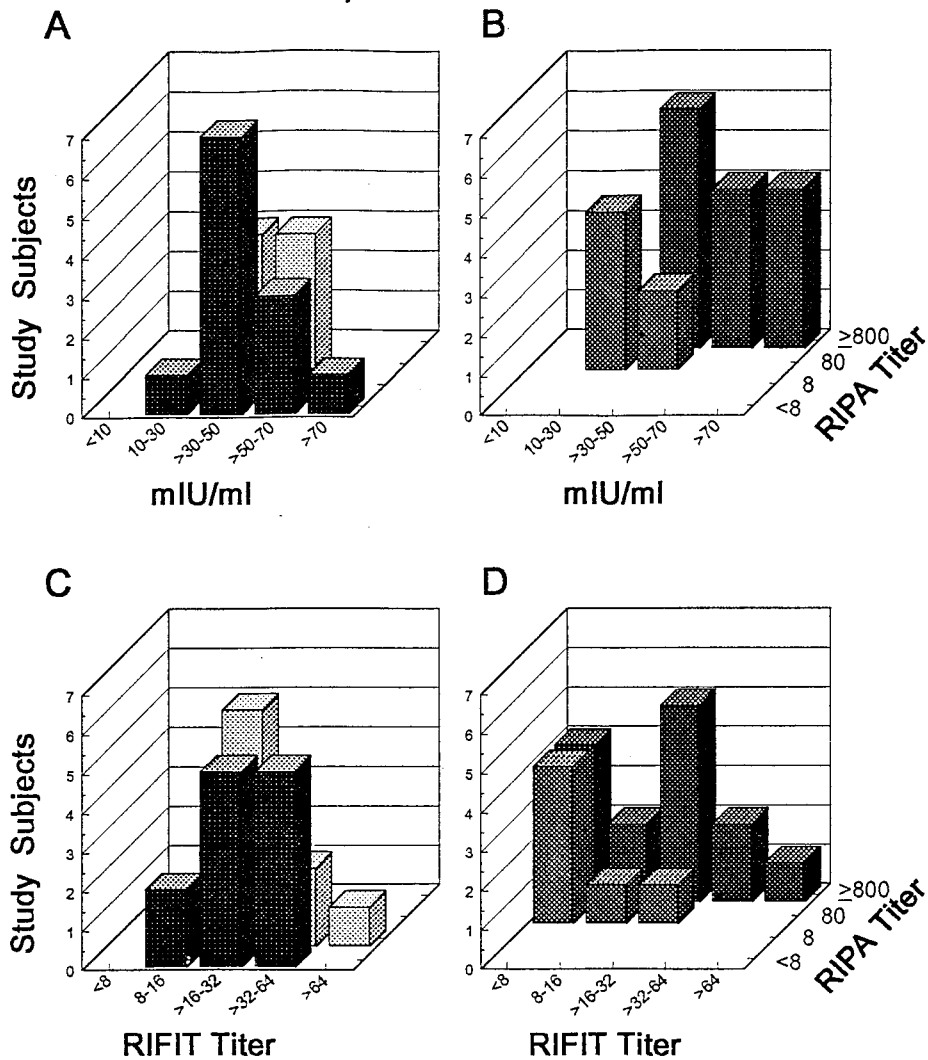


Figure 4. Comparison of modified HAVAB (panels A and B) and RIFIT (panels C and D) measurements of anti-HAV activity with RIPA titers in sera from study subjects bled 7 days after administration of immune globulin (0.06 ml/kg) (panels A and C) or 4 weeks after administration of a single dose of inactivated HAV vaccine (MSD-023) (panels B and D).